elf atochem

ATO

Elf Atochem North America, Inc.
2000 Market Street
Philadelphia, PA 19103-3222
Tel.: 215.419.7000

8EHQ-0797-13985

July 28, 1997

UPS NEXT DAY DELIVERY

Document Control Office (7407)
Office of Pollution Prevention and Toxics
Environmental Protection Agency
401 M St., S.W.
Washington, D.C. 20460

Subject: TSCA Section 8(e) Submission

Contains No CBI

Dear Sir/Madam:

Elf Atochem North America, Inc. (Elf Atochem) has received the final report of a gene mutation test and is submitting this report to the Environmental Protection Agency (EPA) pursuant to Toxic Substances Control Act (TSCA) Section 8(e). This study provides information on n-propyl bromide, CAS number 106-94-5, and does not involve effects in humans.

Nothing in this letter or the enclosed study report is considered confidential business information of Elf Atochem.

The *in vitro* potential of test substance n-propyl bromide to induce mutations at the TK locus in L5178Y mouse lymphoma cells was evaluated in this study. The test substance induced a reproducible and signficant increase in the mutation frequency and the number of small colonies at dose levels between 1000 and 1500 μg/ml without activation and at levels between 1500 and 2000 μg/ml with activation, giving a positive response under the conditions of this test.

This new information adds to the existing genotoxicity data base for this material which includes: positive and negative responses in the Ames Salmonella assay, negative results in the mouse micronucleus assay and the rat dominant lethal assay, and weak positive results in a study for chromosomal aberrations. Results from this study have been incorporated into the Elf Atochem Material Safety Data Sheet for the test material.

Based on EPA guidance we believe that these data meet EPA criteria for reporting under TSCA Section 8(e).

Further questions regarding this submission may be directed to me at (215) 419-5890.

Sincerely,

Debra Randall

Debra Randall, DABT
Product Safety Manager

SPONSOR

Elf Atochem S.A.
Direction Sécurité Environnement - Produit
La Défense 10 - Cédex 42
92091 Paris-la-Défense
France

STUDY TITLE

***IN VITRO* MAMMALIAN CELL GENE MUTATION TEST IN L5178Y TK$^{+/-}$ MOUSE LYMPHOMA CELLS**

TEST SUBSTANCE

n-PROPYL BROMIDE

STUDY DIRECTOR

Brigitte Molinier

STUDY COMPLETION DATE

12th February 1996

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

13293 MLY

CONTENTS

STATEMENT OF THE STUDY DIRECTOR

The study was performed in compliance with the principles of Good Laboratory Practice Regulations:

. Décret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Ministère de l'Industrie et de l'Aménagement du Territoire),
. US Environmental Protection Agency, Federal Register, 40 CFR Part 792; Toxic Substances Control Act; Good Laboratory Practice Standards, November 29, 1983 (and subsequent amendments),
. O.E.C.D. Principles of Good Laboratory Practice, C(81)30(final) Annex 2. May 12, 1981.

I declare that this report constitutes a true and faithful record of the procedures undertaken and the results obtained during the performance of the study.

This study was performed at the Centre International de Toxicologie (C.I.T.), Miserey, 27005 Evreux, France.

B. Molinier Date: 12.2.96
Study Director
Doctor of Toxicology
Head of Genetic Toxicology

STATEMENT OF QUALITY ASSURANCE UNIT

Type of inspections	Dates (day/month/year)		
	Inspections	Report to Study Director (*)	Report to Management (*)
Protocol	25.7.95	1.8.95	1.8.95
Study	11.10.95	16.10.95	18.10.95
Study	2.11.95	3.11.95	6.11.95
Report	24.1.96	26.1.96	28.1.96

The inspections were performed in compliance with C.I.T. Quality Assurance Unit procedures and the Good Laboratory Practice Regulations.

(*) The dates mentioned correspond to the dates of signature of audit reports by Study Director and Management.

L. Valette-Talbi Date: 12.2.96
Doctor of Biochemistry
Head of Quality Assurance Unit
and Scientific Archives

SUMMARY

The objective of this study was to evaluate the potential of the test substance n-PROPYL BROMIDE to induce mutations at the TK (thymidine kinase) locus in L5178Y mouse lymphoma cells.

Methods

After a preliminary toxicity test, n-PROPYL BROMIDE was tested in two independent experiments, with or without a metabolic activation system, the S9 mix, prepared from a liver microsomal fraction (S9 fraction) of rats induced with Aroclor 1254.
Approximately $0.5.10^6$ cells/ml in 20 ml culture medium with 5% horse serum were exposed to the test or control substances, in the presence or absence of S9 mix (final concentration of S9 fraction 2%), for three hours at 37°C.
Cytotoxicity was then determined using cloning efficiency (CE_0) before expression of the mutant phenotype. Cells viability (using cloning efficiency CE_2) and number of mutant clones (differentiating small and large colonies) were checked after the expression of the mutant phenotype.

The test substance was dissolved in dimethylsulfoxide (DMSO).

The dose-levels for the positive controls were as follows:
. without S9 mix: 25 µg/ml of methylmethane sulfonate
. with S9 mix: 3 µg/ml of cyclophosphamide.

Results

The cloning efficiencies CE_0 and CE_2 and mutation frequencies of the vehicle and positive controls were within the range of our historical data.

The top dose-level was selected according to the criteria specified in the international regulations. Since the test substance was toxic, the top dose-level was based on the level of toxicity: a toxic dose-level giving 10-20% relative survival assessed by relative cloning effiency determined on day 0 post-treatment (RCE_0).

Without S9 mix, the selected treatment-levels were:
. first experiment: 125, 250, 500, 1000 and 1500 µg/ml,
. second experiment: 250, 500, 1000, 1250 and 1500 µg/ml.

The test substance induced a reproducible and significant increase in the mutation frequency together with an increase in the number of small colonies at dose-levels between 1000 and 1500 µg/ml.

With S9 mix, the selected treatment-levels were:
. first experiment: 125, 250, 500, 1000, 1500 and 2000 µg/ml,
. second experiment: 500, 1000, 1500, 2000 and 2500 µg/ml. (At 2500 µg/ml, all the cells were dead three hours after treatment).

The test substance did not induce any significant increase in the mutation frequency in the first experiment. In the second one, it induced a significant increase in the mutation frequency together with an increase in the number of small colonies at 1500 and 2000 µg/ml.

Conclusion

Under our experimental conditions, the test substance n-PROPYL BROMIDE showed mutagenic activity in this mouse lymphoma assay, especially without S9 mix.

1. INTRODUCTION

This study was performed at the request of Elf Atochem S.A., Paris-la-Défense, France.

The objective of this study was to evaluate the potential of the test substance to induce mutation at the TK (thymidine kinase) locus in L5178Y mouse lymphoma cells.

This *in vitro* mammalian cell gene mutation test is able to identify substances that cause base-pair mutations, frameshift mutations, small deletions, large deletions and rearrangements of the relevant chromosomes. Mutagenic substances can induce forward mutation from the parental type ($TK^{+/-}$) to the mutant form ($TK^{-/-}$) permitting mutant cells to grow and form colonies in selective medium, while non-mutant cells cannot. In addition, two types of colonies are produced: small, slow growing colonies mainly produced by chromosome rearrangements and large colonies mainly produced by point mutations.

This study was designed in accordance with the following guidelines:
. O.E.C.D. guideline No. 476, 4th April 1984 and revised Draft document of December 1994,
. E.E.C. Directive No. 87/302/E.E.C., 18th November 1987,
. U.S.A./E.P.A./T.S.C.A. Federal Register, Vol. 50, No. 188, Subpart F, 27th September 1985.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Identification

2.1.1.1 Test substance

The test substance, n-PROPYL BROMIDE, used in the study was supplied by Elf Atochem.
Documentation supplied by the Sponsor identified the test substance as follows:
. denomination:
  - protocol and labelling: n-PROPYL BROMIDE
. batch number:
  - protocol: 3-337-02
  - labelling: 3-337-02 CAL 4318/94
. description: colourless liquid
. container: one glass flask
. date of receipt: 4.10.94
. storage conditions: at room temperature and kept from light
. purity: 99.3%.

Data relating to the characterization of the test substance are documented in an analytical certificate and a test article description (presented in section 6) provided by the Sponsor.

2.1.1.2 Vehicle

The vehicle was dimethylsulfoxide (DMSO, batch No. 313K19492750, Merck Clévenot, 77500 Chelles, France).

2.1.2 Preparation

The test substance was dissolved in the vehicle at concentrations between 300 and 500 mg/ml.
The preparations were made immediately before use.

2.1.3 Positive controls

Two known mutagens, dissolved in distilled water, were used to check the sensitivity of the test system:
. without S9 mix: methylmethane sulfonate (MMS), used at a final concentration of 25 µg/ml,
. with S9 mix: cyclophosphamide (CPA), used at a final concentration of 3 µg/ml.

2.2. TEST SYSTEM

2.2.1 Cells

L5178Y cells are an established cell line recommended by international regulations for *in vitro* mammalian cell gene mutation test. They have demonstrated sensitivity to chemical mutagens, a high cloning efficiency and a stable spontaneous mutant frequency. The average cell cycle time is 12-14 hours and the TK phenotypic expression time is two days. Regular checks demonstrated the absence of mycoplasma contamination.

L5178Y cells, originally obtained through ATCC, were kindly supplied by Dr. Oudelkhim-Diot (Vitry-sur-Seine, France).

The cells were stored in a cryoprotective medium (10% horse serum and 10% dimethylsulfoxide (DMSO)) at -80°C.

2.2.2 Metabolic activation system

The S9 mix consists of induced enzymatic systems contained in rat liver microsomal fraction (S9 fraction) and the cofactors necessary for their function.

S9 fraction was purchased from Moltox (Molecular Toxicology, INC, Annapolis, MD 21401, U.S.A.) and obtained from the liver of rats treated with Aroclor 1254 (500 mg/kg) by intraperitoneal route.

The S9 fraction was preserved in sterile tubes within a liquid nitrogen container, until use.
The S9 mix was prepared at +4°C immediately before use and maintained at this temperature until added to culture medium.

The S9 mix contained per 5 ml:
. 1 ml Glucose-6-Phosphate (180 mg/ml),
. 1 ml NADP (25 mg/ml),
. 1 ml KCl (150 mM),
. 2 ml S9 fraction (final concentration of S9 fraction in the S9 mix: 40%, v/v), (batch Nos. 588 and 0616, protein concentration: 37 and 38 g/l, respectively).

2.3. EXPERIMENTAL DESIGN

2.3.1 Treatment

The day before treatment, the cells were seeded in 50 ml of RPMI 1640 medium containing 10% horse serum, L-Glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and sodium pyruvate (200 µg/ml). The cells were then incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. After 24-hour incubation, culture medium was removed and cells were counted.
Approximately 0.5 10^6 cells/ml in 20 ml culture medium with 5% horse serum, were exposed to the test or control substances, in the presence or absence of S9 mix (final concentration of S9 fraction: 2%), for three hours at 37°C.

The treatment medium was then removed and the cells were counted and seeded as follows:

Cytotoxicity plates
1.6 cells/well (one 96-well plate/culture = two plates/dose-level) to determine cytotoxicity using cloning efficiency (CE_0). After 12 ± 1 days of incubation at 37°C, the clones were counted.

To enable the expression of the mutant phenotype, 2.10^5 cells/ml were reincubated at 37°C and replated on day 1 at 2.10^5 cells/ml. After two days, the cells were seeded as follows:

Viability plates
1.6 cells/well (one 96-well plate/culture = two plates/dose-level) to define the number of viable cells (CE_2). After 12 ± 1 days of incubation at 37°C, the clones were counted.

Mutant plates
2000 cells/well (two 96-well plates/culture = four plates/dose-level) to select the TFT^R (trifluorothymidine resistant) mutant cells (for determination of CE_{mutant}). After 12 ± 1 days of incubation at 37°C in the presence of 4 µg TFT/ml of culture medium, the clones were counted, differentiating small and large colonies:
- size of small colonies, < 25% of the diameter of the well,
- size of large colonies, > 25% of the diameter of the well.

2.3.2 Preliminary toxicity test

To assess the cytotoxicity of the test substance, at least six dose-levels of n-PROPYL BROMIDE (one culture/dose-level) were tested both with and without metabolic activation.

2.3.3 Mutagenicity experiments

In two independent experiments, at least four dose-levels of n-PROPYL BROMIDE (two cultures/dose-level) were tested both with and without metabolic activation.
In each experiment, the following controls were included using at least duplicate cultures:
. vehicle controls: cultures treated with the vehicle,
. positive controls: cultures treated with:
- MMS, in the absence of S9 mix,
- CPA, in the presence of S9 mix.

2.4. EVALUATION OF THE RESULTS

Treatment of results

Data from cytotoxicity plates (empty wells) are used to calculate CE_0 (cloning efficiency after treatment) and RCE_0 (survival relative to vehicle controls after treatment). These values give a measure of the toxicity of the treatment with the test substance. CE_0 is calculated from the zero term of the Poisson distribution:

$$CE_0 = \frac{-\text{Ln}\left[\frac{\text{empty wells}}{\text{total wells}}\right]}{\text{number of cells/well (~ 1.6)}}$$

$$RCE_0 = \frac{CE_0 \text{ treated}}{CE_0 \text{ vehicle control}} \times 100$$

Data from viability plates (empty wells) are used to calculate CE_2 (cloning efficiency at the end of the expression period) and RCE_2 (viability relative to vehicle controls at the end of the expression period). These values indicate the viability of the cell populations at the end of the expression period. CE_2 is calculated from the zero term of the Poisson distribution:

$$CE_2 = \frac{-\text{Ln}\left[\frac{\text{empty wells}}{\text{total wells}}\right]}{\text{number of cells/well (~ 1.6)}}$$

$$RCE_2 = \frac{CE_2 \text{ treated}}{CE_2 \text{ vehicle control}} \times 100$$

Data from the mutant plates (empty wells) are used to calculate CE_{mutant} (cloning efficiency in selective medium). This value is an indicator of the absolute mutant frequency. CE_{mutant} is calculated from the zero term of the Poisson distribution (only mutant clones are able to grow in TFT containing medium):

$$CE_{mutant} = \frac{-\text{Ln}\left[\frac{\text{empty wells}}{\text{total wells}}\right]}{\text{number of cells/well (~ 2000)}}$$

The relative mutant frequency (MF) is calculated as:

$$MF = \frac{CE_{mutant} \times 10^6}{CE_2}$$

For each experimental point, the scored values (empty wells), the number of small colonies, CE_0, RCE_0, CE_2, RCE_2 and MF will be presented in a table.

Acceptance criteria

This study was considered valid since the following criteria were fulfilled:
. the cloning efficiency of the vehicle controls were between 0.6-1.4 for CE_0 and between 0.7-1.3 for CE_2,
. the mutation frequency of the vehicle controls were between 60-200.10^{-6},
. the mutation frequency of the positive controls was higher than that of the vehicle controls and within the range of our historical data.

Evaluation criteria

A reproducible two-fold increase in the mutant frequency when compared with the vehicle controls, at any dose-level and/or evidence a dose-relationship were considered as a positive result. Reference to historical data, or other considerations of biological relevance might be also taken into account in the evaluation of the data obtained.

2.5. ARCHIVES

The study documentation and materials, namely:
. protocol and possible amendments,
. raw data,
. correspondence,
. final report and possible amendments,
. a sample of the test substance,

are stored in the archives of C.I.T. (Miserey, 27005 Evreux, France), for five years after the end of the experimental study. At the end of this period, the study documentation will be returned to the Sponsor.

2.6. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedure	Date
Protocol approved by:	
. Study Director	5.7.95
. Sponsor	24.7.95
Preliminary toxicity test with and without S9 mix	
. treatment	13.9.95
First experiment without S9 mix	
. treatment	11.10.95
with S9 mix	
. treatment	17.10.95
Second experiment without and with S9 mix	
. treatment	31.10.95

3. RESULTS (tables 1 to 5)

3.1. PRELIMINARY TOXICITY TEST (table 1)

The test substance was freely soluble in the vehicle (DMSO) at 500 mg/ml. Thereafter, a moderate to strong emulsion was observed in the culture medium at the final dose-levels of 2500 to 5000 µg/ml. Consequently, the maximum dose-level was 2500 µg/ml.

The treatment-levels were: 10, 25, 100, 250, 1000 and 2500 µg/ml.

Marked cytotoxicity of the test substance was recorded at 2500 µg/ml: all the cells were dead after three hours of treatment both with and without S9 mix. At the lower dose-levels, a moderate reduction of the cloning efficiency CE_0 of treated cultures when compared to control cultures was observed.

3.2. MUTAGENICITY EXPERIMENTS (tables 2 to 5)

The cloning efficiencies CE_0 and CE_2 and the mutation frequencies of the vehicle and positive controls were as specified in acceptance criteria and within the range of our historical data.

The top dose-level was selected according to the criteria specified in the international regulations. Since the test substance was toxic, the top dose-level was based on the level of toxicity: a toxic dose-level giving 10-20% relative survival assessed by relative cloning effiency determined on day 0 post-treatment (RCE_0).

Without S9 mix, the selected treatment-levels were:
. first experiment: 125, 250, 500, 1000 and 1500 µg/ml,
. second experiment: 250, 500, 1000, 1250 and 1500 µg/ml.

At 1500 µg/ml, 21 and 33% RCE_0 were noted when compared to control cultures for the first and second experiments, respectively.
At 1250 µg/ml (second experiment), RCE_0 was 46%.
At lower dose-levels, no significant differences between treated and control were noted.

The number of viable cells two days after treatment (assessed by the cloning efficiencies CE_2) was considered equivalent to that of the control cultures.

The test substance induced a reproducible and significant increase in the mutation frequency together with an increase in the number of small colonies at dose-levels between 1000 and 1500 µg/ml.

With S9 mix, the selected treatment-levels were:
. first experiment: 125, 250, 500, 1000, 1500 and 2000 µg/ml,
. second experiment: 500, 1000, 1500, 2000 and 2500 µg/ml.

No significant cytotoxicity was observed in the first experiment, only a moderate reduction of the RCE_0 (59%) was noted at 2000 µg/ml. In the second experiment, cytotoxicity was more marked:
At 2500 µg/ml, all the cells were dead three hours after treatment.
At 2000 µg/ml, RCE_0 was 9%.
At 1500 µg/ml, RCE_0 was 36%.
At lower dose-levels, no marked differences between treated and control were noted.

The number of viable cells two days after treatment (assessed by the cloning efficiencies CE_2) was considered equivalent to that of the control cultures.

The test substance did not induce any significant increase in the mutation frequency in the first experiment. In the second one, it induced a significant increase in the mutation frequency together with an increase in the number of small colonies at 1500 and 2000 µg/ml.

4. CONCLUSION

Under our experimental conditions, the test substance n-PROPYL BROMIDE showed mutagenic activity in this mouse lymphoma assay, especially without S9 mix.

5. REFERENCES

Nestmann, E.R.; Brillinger, R.L.; Gilman, J.P.W.; Rudd, C.J. and Swierenga, S.H.H.: Recommended protocols based on a survey of current practice in genotoxicity testing Laboratories: II. Mutation in Chinese hamster ovary, L5178Y Chinese hamster lung and L5178 mouse lymphoma cells. Mutation Research, 246, 255-284 (1991).

Ames, B. N.; Mc Cann, D. and Yamasaki, E.: Methods for detecting carcinogens and mutagens with the *Salmonella* Mammalian-microsome mutagenicity test. Mutation Research, 31, 347-364 (1975).

Table 1: Preliminary toxicity test

	Doses (µg/ml)	Empty wells	Total wells	CEo	RCEo %
	0	1	96	2.85	100
without	10	7	96	1.64	57
S9 mix	25	5	96	1.85	65
	100	2	96	2.42	85
	250	5	96	1.85	65
	1000	10	96	1.41	50
	2500	-	96	-	-
	0	2	96	2.42	100
with	10	4	96	1.99	82
S9 mix	25	4	96	1.99	82
	100	4	96	1.99	82
	250	5	96	1.85	76
	1000	8	96	1.55	64
	2500	-	96	-	-

- : no seeding of CEo performed since all cells died after 3 hours of exposure to the test substance
0 : vehicle control (DMSO)
CEo: cloning efficiency
RCEo : relative cloning efficiency

Table 2: First experiment without S9 mix

	Cytotoxicity: CEo				Viable cells: CE2				Mutation frequency: MF				
Doses (µg/ml)	Empty wells	Total wells	CEo	RCEo %	Empty wells	Total wells	CE2	RCE2 %	Nb. SC	Empty wells	Total wells	MF	R
	34	96			18	96			5	77	96		
0			0.66	100			0.92	100	4	78	96	122	1.0
	33	96			26	96			6	76	96		
									6	76	96		
	20	96			29	96			6	73	96		
125			0.94	142			0.77	84	7	75	96	156	1.3
	23	96			27	96			6	78	96		
									7	76	96		
	31	96			22	96			7	72	96		
250			0.87	132			0.78	85	4	75	96	158	1.3
	17	96			33	96			5	78	96		
									10	75	96		
	32	96			30	96			8	72	96		
500			0.72	109			0.85	93	7	80	96	143	1.2
	29	96			19	96			2	73	96		
									5	76	96		
	38	96			29	96			8	73	96		
1000			0.74	112			0.73	79	12	62	96	250	2.1
	21	96			31	96			15	66	96		
									12	66	96		
	71	96			27	96			29	41	96		
1500			0.14	21			0.79	86	29	53	96	514	4.2
	83	96			27	96			43	37	96		
									32	39	96		
	35	96			55	96			23	52	96		
MMS			0.64	97			0.41	44	27	44	96	856	7.0
25	34	96			45	96			30	49	96		
									27	46	96		

CEo and CE2: cloning efficiency

RCE(o and 2): relative cloning efficiency

SC: small colonies

R: Ratio between Mutation Frequency of treated cells/Mutation Frequency of control cells

Table 3: First experiment with S9 mix

	Cytotoxicity: CEo				Viable cells: CE2				Mutation frequency: MF				
Doses (µg/ml)	Empty wells	Total wells	CEo	RCEo %	Empty wells	Total wells	CE2	RCE2 %	Nb. SC	Empty wells	Total wells	MF	R
	24	96			33	96			5	76	96		
0			0.79	100			0.79	100	5	77	96	162	1.0
	30	96			21	96			6	74	96		
									3	70	96		
	17	96			29	96			5	76	96		
125			1.03	130			0.82	103	7	78	96	135	0.8
	20	96			23	96			6	75	96		
									6	79	96		
	26	96			25	96			8	76	96		
250			0.83	105			0.74	93	5	75	96	176	1.1
	25	96			34	96			6	72	96		
									3	73	96		
	28	96			23	96			7	65	96		
500			0.75	94			0.96	122	3	75	96	154	1.0
	30	96			18	96			6	73	96		
									8	72	96		
	44	96			20	96			5	70	96		
1000			0.55	69			0.87	109	11	66	96	191	1.2
	36	96			28	96			9	72	96		
									10	68	96		
	39	96			28	96			9	73	96		
1500			0.67	84			0.70	88	9	69	96	245	1.5
	27	96			35	96			8	68	96		
									8	63	96		
	41	96			27	96			9	76	96		
2000			0.47	59			0.73	92	9	74	96	149	0.9
	50	96			33	96			2	84	96		
									8	75	96		
	52	96			55	96			36	40	96		
CPA			0.36	45			0.33	42	35	38	96	1284	7.9
3	56	96			58	96			31	44	96		
									34	42	96		

CEo and CE2: cloning efficiency

RCE(o and 2): relative cloning efficiency

SC: small colonies

R: Ratio between Mutation Frequency of treated cells/Mutation Frequency of control cells

Table 4: Second experiment without S9 mix

Cytotoxicity: CEo					Viable cells: CE2				Mutation frequency: MF				
Doses (µg/ml)	Empty wells	Total wells	CEo	RCEo %	Empty wells	Total wells	CE2	RCE2 %	Nb. SC	Empty wells	Total wells	MF	R
	30	96			32	96			4	83	96		
0			0.80	100			0.80	100	6	76	96	147	1.0
	23	96			21	96			5	71	96		
									8	73	96		
	35	96			21	96			2	78	96		
250			0.74	92			0.92	114	0	75	96	122	0.8
	24	96			23	96			4	77	96		
									5	77	96		
	12	96			27	96			2	76	96		
500			1.14	142			0.85	106	2	82	96	133	0.9
	19	96			22	96			6	74	96		
									4	74	96		
	34	96			28	96			5	73	96		
1000			0.64	80			0.87	108	8	62	96	239	1.6
	35	96			20	96			10	60	96		
									11	59	96		
	46	96			26	96			14	62	96		
1250			0.37	46			0.84	105	12	67	96	275	1.9
	60	96			24	96			16	56	96		
									18	57	96		
	56	96			18	96			22	53	96		
1500			0.26	33			0.88	109	28	42	96	424	2.9
	70	96			29	96			30	46	96		
									33	41	96		
	39	96			83	96			22	54	96		
MMS			0.71	88			0.22	27	23	59	96	1467	10.0
25	23	96			53	96			32	45	96		
									32	46	96		

CEo and CE2: cloning efficiency

RCE(o and 2): relative cloning efficiency

SC: small colonies

R: Ratio between Mutation Frequency of treated cells/Mutation Frequency of control cells

Table 5: Second experiment with S9 mix

Cytotoxicity: CEo					Viable cells: CE2				Mutation frequency: MF				
Doses (µg/ml)	Empty wells	Total wells	CEo	RCEo %	Empty wells	Total wells	CE2	RCE2 %	Nb. SC	Empty wells	Total wells	MF	R
	27	96			29	96			4	78	96		
0			0.83	100			0.83	100	6	75	96	123	1.0
	24	96			22	96			4	79	96		
									2	81	96		
	32	96			19	96			5	71	96		
500			0.60	72			1.03	124	3	77	96	125	1.0
	42	96			18	96			9	71	96		
									2	78	96		
	41	96			33	96			4	67	96		
1000			0.60	73			0.78	94	7	79	96	200	1.6
	32	96			22	96			6	69	96		
									7	66	96		
	55	96			21	96			23	56	96		
1500			0.30	36			0.85	103	19	54	96	316	2.6
	64	96			28	96			15	57	96		
									11	57	96		
	86	96			39	96			29	46	96		
2000			0.07	9			0.63	76	-	-	-	631	5.1
	85	96			31	96			20	40	96		
									17	44	96		
	60	96			49	96			54	19	96		
CPA			0.31	38			0.43	52	49	28	96	1433	11.6
3	56	96			48	96			38	37	96		
									43	29	96		

CEo and CE2: cloning efficiency

RCE(o and 2): relative cloning efficiency

SC: small colonies

R: Ratio between Mutation Frequency of treated cells/Mutation Frequency of control cells

-: due to the toxicity of the test substance, there were not enough cells to allow seeding of this duplicate

APPENDICES

1. Analytical certificate of the test substance and test article description

elf atochem
ATO
Port-de-Bouc/Fos

Port de Bouc, le 31-08-1994

CERTIFICAT D'ANALYSE EXPEDITION

Nature du produit : BROMURE DE PROPYLE

Date de l'expedition : 11-08-94

Destinataire : CAL

Transport : 1 kg

Lot N° : 3-337-02

Analyse :

RP	: 99.3	%
Alcool	: 0.01	%
Chlorure	: 0.07	%
BIP	: 0.16	%
Ether	: 0.35	%
Lourds	: 0.04	%

Le Responsable Laboratoire
REYNAUD

TOXICOLOGY DEPARTMENT
CONFIDENTIAL
7 September 1994

elf atochem s.a.
La défense 10, cedex 42
92091 Paris-la-Défense, France

TEST ARTICLE DESCRIPTION

n-PROPYL BROMIDE

STRUCTURAL FORMULA

$BrCH_2-CH_2-CH_3$

IDENTITY

Test article name	:	n-Propyl bromide
Chemical name	:	Bromo-1-propane
CAS number	:	106-94-5
Molecular formula	:	BrC_3H_7
Molecular weight	:	123
Purity	:	99.3 %
Origin and batch	:	Elf Atochem, Port-de-Bouc, batch 3-337-02
Elf Atochem filing number	:	CAL 4318/94

PHYSICAL AND CHEMICAL PROPERTIES

Appearance	:	Colorless to clear yellow liquid
Specific gravity	:	1.353 at 20°C
Melting point	:	-110°C
Boiling point	:	71°C
Vapor pressure	:	146 mbar at 20°C
Flash point	:	<21°C (closed cup)
Solubility	:	0.25 % in water at 20°C
	:	soluble in DMSO
	:	soluble in ethylic alcohol

TOXICOLOGICAL INFORMATIONS AND USE SAFETY

LD_{50}/Oral/Rat > 2000 mg/kg. Irritant to the skin and eyes.

STORAGE AND DISPOSAL

Storage	:	In dark and at room temperature
Expiry date	:	December 1995
Disposal	:	Incineration

2. Historical data

MLY 7.2.95

Historical data

Mouse lymphoma assay

	without S9 mix						with S9 mix					
	vehicle			MMS: 25 µg/ml			vehicle			CPA: 30 µg/ml		
assay No.	CE0	CE2	MF	CE0	CE2	MF	CE0	CE2	MF	CE0	CE2	MF
1	0.58	1.07	126	0.49	1.02	458	0.62	1.12	131	0.39	0.99	457
2	0.65	0.74	135	0.36	0.43	945	0.75	0.91	182	0.4	0.97	442
3	0.78	1.27	109	1.02	0.60	754	0.93	0.58	235	0.76	0.61	610
4	0.62	1.29	93	0.55	0.67	413	0.78	0.96	128	0.62	0.76	453
5	0.91	0.90	90	0.84	0.46	806	0.58	0.80	105	0.35	0.4	812
6	0.69	1.73	81	0.71	0.96	399						
7	0.95	0.95	85	1.13	0.73	511						
8	0.50	0.55	207	0.51	0.46	625	0.87	0.93	131	0.53	0.6	638
9	0.63	0.57	236	0.59	0.57	705	0.65	1.48	99	0.42	0.57	642
10	1.39	1.21	65	0.76	0.84	396	1.59	1.73	48	0.56	0.73	384
11	1.43	1.04	73	1.35	0.62	445	0.82	1.82	45	0.59	0.95	386
12	0.73	1.73	40	0.95	0.74	387	0.66	0.88	59	0.5	0.53	380
13	0.58	0.82	107	0.34	0.32	650	0.74	0.88	98	0.56	0.7	224
14	0.82	0.90	49	0.45	0.78	330	0.57	0.80	161	0.4	0.48	565
15	0.63	0.51	169	0.45	0.34	884	0.76	0.74	193	0.17	0.24	1804
16	0.60	0.68	60	0.21	0.36	452	0.67	0.85	139	0.17	0.23	1513
17	0.65	0.93	184	0.63	0.45	775	0.43	0.74	177	0.04	0.16	1520
18	0.61	0.55	202	0.50	0.47	800	0.64	0.73	177	0.09	0.16	1974
19	0.82	0.79	121	0.65	0.48	771	0.68	0.66	134	0.25	0.38	1057
20	0.65	0.65	94	0.70	0.48	565	0.63	0.59	129	0.1	0.17	1819
21	0.56	0.83	77	0.57	0.65	454	0.75	0.62	84	0.48	0.41	758
minimum	0.50	0.51	40	0.21	0.32	330	0.43	0.58	45	0.04	0.16	224
maximum	1.43	1.73	236	1.35	1.02	945	1.59	1.82	235	0.76	0.99	1974

CE0: Cytotoxicity: cloning efficiency at the end of the treatment period
CE2: Viability: cloning efficiency at the time of plating in selective conditions
MF: mutant frequency: number of mutant cells divided by the number of viable cells
Vehicle: DMSO, culture medium or distilled water
MMS: methylmethane sulfonate
CPA: cyclophosphamide

3. Protocol and amendment

C I T

centre international de toxicologie

MISEREY BP 563 27005 EVREUX CEDEX FRANCE TÉL. 32 29 26 26 TÉLÉCOPIE 32 67 87 05

Miserey, 5th July 1995

n-PROPYL BROMIDE

IN VITRO MAMMALIAN CELL GENE MUTATION TEST TK/L5178Y MOUSE LYMPHOMA

Protocol from	:	Centre International de Toxicologie Miserey BP 563 - 27005 Evreux Cédex France
Sponsor	:	Elf Atochem S.A.
Address	:	La Défense 10, Cédex 42 92091 Paris-la-Défense France
Study Monitor	:	J.F. Régnier
Study Director	:	B. Molinier
Study Number	:	13293 MLY

INTRODUCTION

The objective of this study is to evaluate the potential of the test substance to induce a gene mutation at the TK (thymidine kinase) locus in L5178Y Mouse lymphoma cells.

The purpose of this *in vitro* mammalian cell gene mutation test is to identify substances that cause base-pair mutations, frameshift mutations, small deletions, large deletions and rearrangements of the relevant chromosomes. Mutagenic substances can induce a forward mutation from the parental type ($TK^{+/-}$) to the mutant form ($TK^{-/-}$) in the cells which are then able to grow and form colonies on a selective medium, while non-mutated cells cannot. In addition, two types of colonies are produced: small, slow growing colonies mainly produced by chromosome rearrangements and large colonies mainly produced by point mutations.

This protocol complies with:

- O.E.C.D. guideline No. 476, 4th April 1984 and revised Draft document of December 1994.
- E.E.C. Directive No. 87/302/E.E.C., 18th November 1987.
- U.S.A./E.P.A./T.S.C.A. Federal Register, Vol. 50, No. 188, Subpart F, 27th September 1985.

The study will be conducted in compliance with the following Good Laboratory Practice regulations:

- Décret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Ministère de l'Industrie et de l'Aménagement du Territoire).
- US Environmental Protection Agency, Federal Register, 40 CFR Part 792; Toxic Substances Control Act; Good Laboratory Practice Standards, November 29, 1983 (and subsequent amendments).
- O.E.C.D. Principles of Good Laboratory Practice, C(81)30(final) Annex 2. May 12, 1981.

MATERIALS AND METHODS

1. TEST AND CONTROL SUBSTANCES

1.1 Identification

1.1.1 Test substance

. **Denomination:** n-PROPYL BROMIDE
. **Batch No.:** 3-337-02
. **Description:** colourless to clear yellow liquid
. **Storage conditions:** in dark and at room temperature
. **Physico-chemical properties:** i.e. purity, composition, stability and expiry date which refer to the batch to be used and handling conditions will be indicated in the test substance data sheet (to be completed by the Sponsor). An analytical certificate will also be provided by the Sponsor.
. **Required amount:** 5 g

1.1.2 Vehicle

The vehicle will be selected according to the results of solubility tests performed before the preliminary toxicity test (1).

1.2 Preparation

The test substance will be dissolved in the vehicle to provide approximately 100 to 400 fold the final dose, depending on the vehicle. The preparations will be made immediately before use.

1.3 Positive controls

Two known mutagens, dissolved in distilled water, will be used to check the sensitivity of the test system:
. without S9 mix: methylmethane sulfonate (MMS), used at 25 µg/ml,
. with S9 mix: cyclophosphamide (CPA), used at 3 µg/ml.

2. TEST SYSTEM

2.1 Cells

L5178Y cells are an established cell line recommended by international regulations for *in vitro* mammalian cell gene mutation test. They have a high cloning efficiency (> 50%), an average cell cycle time of 12-14 hours and a TK phenotypic expression time of two days. They are checked periodically for mycoplasma contaminations.
They are stored in a cryoprotective medium containing 10% horse serum and 10% dimethylsulfoxide (DMSO) at -80°C.

1) To be specified, dated and signed by the Sponsor

2.2 Metabolic activation system: S9 mix

The S9 mix consists of induced enzymatic systems contained in rat liver microsomal fractions (S9) and the cofactors necessary for their function. S9 will be prepared at C.I.T. from the liver of rats treated with Aroclor 1254 (500 mg/kg) by the intraperitoneal route. The S9 fraction is preserved in sterile tubes within a liquid nitrogen container, until use.

The S9 mix will be prepared at +4°C immediately before use and will be maintained at +4°C throughout the experiment.

The S9 mix will contain per 5 ml:
. 1 ml Glucose-6-Phosphate (180 mg/ml),
. 1 ml NADP (25 mg/ml),
. 1 ml KCl (150 mM),
. 2 ml S9 (therefore final concentration of S9 in the S9 mix will be 40% (v/v)).

3. EXPERIMENTAL DESIGN

3.1 Rationale for dose selection

The top dose will be selected according to the following criteria specified in international regulations:
. pH shifts of no more than one pH unit when compared to vehicle control medium,
. osmolality increase of no more than 100 mosm/kg H_2O when compared to vehicle control culture medium,
. for non-toxic freely soluble test substances, the top dose will be 10 mM or 5000 µg/ml,
. for poorly soluble test substances, the top dose will be a dose above the limit of solubility in the final culture medium at the end of the treatment period,
. for toxic test substances, the top dose will be a toxic dose giving 10-20% relative survival relative cloning efficiency determined on day 0 post-treatment. However, this top dose will not exceed the limit of solubility in the final culture medium at the end of the treatment period.

3.2 Treatment

The day before treatment, the cells will be seeded in 50 ml of RPMI 1640 medium containing 10% horse serum, L-Glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and sodium pyruvate (200 µg/ml) in flasks. The flasks will then be placed at 37°C in a humidified atmosphere of 5% CO_2/95% air for 24 hours. The culture medium will then be removed and cells will be counted. Approximately 0.5 10^6 cells will be exposed to the test or control substances, with or without S9 mix, for three hours at 37°C in the culture medium with 5% horse serum (containing 2% S9, for treatment with S9 mix).

The treatment medium will then be removed and the cells will be counted and seeded as follows:

. 1.6 cells/well (one 96-well plate/culture = two plates/dose) to determine cytotoxicity using cloning efficiency (CEo). After 12 ± 1 days of incubation at 37°C, the clones will be counted.

. 2.10^5 cells/ml will be reincubated at 37°C into flasks for two days tc enable the expression of the mutant phenotype. They will be replated on day 1 at 2.10^5 cells/ml.

After these two days, the cells will be seeded as follows:

. 1.6 cells/well (one 96-well plate/culture = two plates/dose) to define the number of viable cells (CE_2). After 12 ± 1 days of incubation at 37°C, the clones will be counted.

. 2000 cells/well (two 96-well plates/culture = four plates/dose) to select the TFT^R (trifluorothymidine resistant) mutant cells. After 12 ± 1 days of incubation at 37°C in the presence of 4 µg TFT/ml of culture medium, the clones will be counted, differentiating small and large colonies:
- size of small colonies < 25% diameter of one well,
- size of large colonies > 25% diameter of one well.

3.2.1 Preliminary toxicity test

To assess the toxicity of the test substance to the cells, at least six doses of the test substance (one culture/dose) will be tested on the L5178Y cells, with or without metabolic activation.

3.2.2 Mutagenicity tests

In two independent tests, at least four doses of the test substance (two cultures/dose) will be tested on the L5178Y cells, with or without metabolic activation.

During each test, the following controls will be made using at least duplicate cultures:
. vehicle control: culture treated with the vehicle,
. positive control: culture treated with:
- MMS, without S9 mix,
- CPA, with S9 mix.

4. EVALUATION OF THE RESULTS

Treatment of results

During each test, for each experimental point, the number of clones corresponding to the cloning efficiencies CEo and CE_2, and the number of TFT^R mutant clones (small and large) will be scored. Individual results corresponding to cytotoxicity (CE_0), viable cells (CE_2), mutation frequency (MF) will be presented in a table.

For each dose, the cloning efficiencies will be calculated from the zero term of the Poisson distribution as follows:

$$CE = \frac{-Ln \frac{\text{empty wells}}{\text{total wells}}}{\text{number of cells per well}}$$

The cytotoxicity of the test substance, CE_0 (%), will be expressed as a percentage related to that of the vehicle controls.

The mutation frequency (MF) will be calculated as follows:

$$MF = \frac{CE \text{ mutant} \times 10^6}{CE_2}$$

with number of cells per well = 1.6 for CE_0 and CE_2 and 2000 for MF

Acceptance criteria

This study will be considered valid if the following criteria are fully met:

. the cloning efficiency of the controls should be between 60 and 140% for CEo and between 70 and 130% for CE_2,

. the mutation frequency of the controls should be between $60\text{-}200.10^{-6}$,

. the mutation frequency of the positive controls should be higher than the controls and within the range of our historical data.

Evaluation criteria

Biological relevance of the results will be considered first. In addition, the following criteria may be used as an aid for determining a positive response:

. a dose-related increase in mutant frequency,

and/or,

. a reproducible increase in the mutant frequency (i.e. at least a doubling when compared to that of the controls) for at least one of the doses.

5. PROCEDURES

The procedures used during the study will be those documented in the relevant C.I.T. procedures manual.

6. AMENDMENTS TO THE PROTOCOL

If necessary, amendments to the protocol will be made after agreement between the Study Director and the Study Monitor.

7. REPORTING

The Study Director will contact the Study Monitor when necessary.
The final report, in English, will contain all data collected throughout the study.
Number of copies of the final report: 5 (4 + 1 unbound) (1)
Proposed issue of the draft report: one month after the end of the study.

8. QUALITY ASSURANCE UNIT

The Quality Assurance Unit will conduct inspections according to Good Laboratory Practice Regulations (as specified on page 2).
The dates on which the findings of these inspections are reported to the Study Director and C.I.T. Management will be specified in the final report.

9. ARCHIVES

The study archives:
. protocol and possible amendments
. raw data
. correspondence
. final report and possible amendments
. sample of the test substance

will be stored on the premises of C.I.T., 27005 Miserey, Evreux, France, for five years after the end of the experimental study. At the end of this period, the study archives will be returned to the Sponsor.

10. TIME SCHEDULE

Beginning of the study: 2nd half of August 1995,
End of the study: 2nd half of October 1995.

(1) Except for special requests made by the Sponsor

Protocol approved by:

J.F. Le Bigot or S. de Jouffrey
C.I.T. Scientific Management
Date: 5.7.95

J.F. Régnier
Study Monitor
Date: 24/7/95

B. Molinier
C.I.T. Study Director
Date: 5.7.95

AMENDMENT TO PROTOCOL

STUDY No.: **13293 MLY**
SPONSOR: **Elf Atochem**
TEST SUBSTANCE: **n-PROPYL BROMIDE**
AMENDMENT No.: 01

Date of application: 13.9.95

MATERIALS AND METHODS

2. TEST SYSTEM

2.2 Metabolic activation system: S9 mix

S9 fraction will be purchased from Moltox (Molecular Toxicology, INC, Annapolis, MD 21401, U.S.A.).

Scientific management	Study Director	Study Monitor
J.F Le Bigot or S. de Jouffrey	B. Molinier	J.F Regnier
Date: 29.1.96	Date: 29.1.96	Date: 6/02/96
Signature:	Signature:	Signature: